REVIEW

Review: Biological and Molecular Differences between Tail Regeneration and Limb Scarring in Lizard: An Inspiring Model Addressing Limb Regeneration in Amniotes

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ABSTRACT

Tissue regeneration in lizards represents a unique model of regeneration and scarring in amniotes. The tail and limb contain putative stem cells but also dedifferentiating cells contribute to regeneration. Following tail amputation, inflammation is low and cell proliferation high, leading to regeneration while the intense inflammation in the limb leads to low proliferation and scarring. FGFs stimulate tail and limb regeneration and are present in the wound epidermis and blastema while they disappear in the limb wound epidermis 2–3 weeks postamputation in the scarring outgrowth. FGFs localize in the tail blastema and the apical epidermal peg (AEP), an epidermal microregion that allows tail growth but is absent in the limb. Inflammatory cells invade the limb blastema and wound epidermis, impeding the formation of an AEP. An embryonic program of growth is activated in the tail, dominated by Wnt-positive and -negative regulators of cell proliferation and noncoding RNAs, that represent the key regenerative genes. The balanced actions of these regulators likely impede the formation of a tumor in the tail tip. Genes for FACIT and fibrillar collagens, protease inhibitors, and embryonic keratins are upregulated in the regenerating tail blastema. A strong downregulation of genes for both B and T-lymphocyte activation suggests the regenerating tail blastema is a temporal immune-tolerated organ, whereas a scarring program is activated in the limb. Wnt inhibitors, pro-inflammatory genes, negative regulators of cell proliferation, downregulation of myogenic genes, proteases, and oxidases favoring scarring are upregulated. The evolution of an efficient immune system may be the main limiting barrier for organ regeneration in amniotes, and the poor regeneration of mammals and birds is associated with the efficiency of their mature immune system. This does not tolerate embryonic antigens formed in reprogrammed embryonic cells (as for neoplastic cells) that are consequently eliminated impeding the regeneration of lost organs. *J. Exp. Zool. (Mol. Dev. Evol.) 00B:1–22, 2017.* © 2017 Wiley Periodicals, Inc.

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BIOLOGICAL CONTEXT AND POTENTIAL FOR TISSUE REGENERATION IN LIZARDS

The evolution of the complexity in the body of vertebrates has improved the control of the internal environment (inner milieu) by neuroendocrine and other mechanisms of feedback for monitoring body temperature, pH, ion concentration, etc. and immunological processes to preserve the bioself against extraneous molecules and microbes. In permanently aquatic vertebrates (fish and some amphibians) and semiaquatic anamniotes (urodele and anuran amphibians), the immune system is mainly innate while the adaptive immune system becomes more effective after metamorphosis (Robert and Cohen, '98; Harty et al., 2003; Danilova, 2006). Fish and amphibians, at least before metamorphosis, conserve a variable and sometimes high degree of regenerative capabilities, unmatched in any amniote (Mesher and Neff, 2006; King et al., 2012). Terrestrial amphibians and some urodels, tend to lose the regenerative capability (Mufti and Simpson, '72; Scadding '77). The relative poor development of the adaptive immune system based on T and B lymphocyte differentiation during development, in addition to the permanence of numerous stem/progenitor cells in the body of anamniotes, particularly in neotenic-like urodele amphibians (Grigoryan, 2016) and relative lower body temperatures are positive conditions that favor tissue healing and organ regeneration. The presence of water on the skin surface reduces the permanence of a low mass of microorganisms penetrating the body, another condition that limits the intervention of immune cells and favors tissue and even organ regeneration.

The transition from an aquatic to a terrestrial environment has required profound transformations not only in the anatomy and physiology but also in the healing capabilities of the organs of amniotes. The risk of an amputation of the tail or limbs in land vertebrates is mainly related to (1) blood and water loss, (2) microbe infection, and (3) loss of motile function that transform a rapid escaping vertebrate into an easy prey. Conversely, in water the fins or limbs are less important than the tail for movement, and their regeneration is compatible with an adaptive pressure so that a regenerative process has evolved (Scadding, '77; Alibardi, 2010a). To survive in the terrestrial environment where the microbial mass is high and microwounds more frequent than in the aquatic world of anamniotes, solar irradiation induces more mutations in the integument, the skin, and the immune system of amniotes which has evolved sophisticated protective mechanisms in addition to innate immunity and can detect an enormous number of extraneous antigens entering the body (Robert and Cohen, '98; Danilova, 2006). The adaptive immune system in amniotes develops progressive antigen-recognition competency as the embryo develops, and cells lose embryonic antigens to acquire those of the adult, after hatching or parturition (Mold and McCune, 2012; Mescher et al., 2013, 2016). This indicates that the surviving immune T- and B-lymphocytes of adult amniotes tolerate adult antigens, but they may be unfit to tolerate embryonic-like antigens that have been altered during development, antigens that largely disappear in tissues by the end of embryogenesis into the adult. In the adult, most of the self-antigens are tolerated and when embryonic antigens are reformed, like in cancer cells or after wounding they may be tagged and eliminated. This is the price endotherm amniotes, such as birds and mammals in particular, has to pay for their efficient control of their "inner milieu": They have become intolerant to embryonic antigens including those of newly reformed embryonic cells after wounding and healing and are consequently biologically unfit to be good regenerators. Amniotes have evolved an efficient body to avoid injury, and when this does occur these vertebrates tend to rapidly isolate the microbial invasion through scarring (Ferguson and O'Kane, 2004). In line with this argument, it is also expected that cell niches (including hemopoietic compartments) are regions with local immune tolerance where mechanisms of immune-evasion are present and allow for a suitable environment to preserve stem cells and the derived proliferating cells (amplifying cells). The study of anuran amphibians and lizards indicates that the key factor involved in the inhibition of organ regeneration in amniotes, including man, is the immune system, and that limiting inflammation and recreating an immune-evasive embryonic environment are the premises for regenerating a new organ. We will discuss in the remaining part of this review this hypothesis after introducing the model of organ regeneration in lizards (Alibardi, 2010a,b, 2014).

FROM ANAMNIOTE REGENERATION TO AMNIOTE SCARRING

On the above premises, the typical reaction to wounds in mammals is a fast healing process aiming to rapidly seal wounds, and this process usually gives rise to a fibrotic or scarring output (Ferguson and O'Kane, 2004). It is possible that the process of scarring evolved in amniotes from the simpler healing of anamniotes, and here we present some thoughts supporting this hypothesis. Amniotes are terrestrial-adapted vertebrates, and they have developed different physiological and anatomical adaptive characteristics to those of anamniotes, so that what we learn from urodele amphibian regeneration can only be partially translated into applications to amniote regeneration. In reptilian amniotes, temperature control is exothermic, varying according to the surrounding environment and is often behaviorally driven (many species bask and after some time they move to a cooler environment). Most reptiles, however, especially during the warmer season when they are active and their immune system nearly as efficient as that of endotherms (Zimmerman et al., 2010), repair wounds by scarring (Bellairs and Bryant, '85; Alibardi, 2010a).

An exception is found in lizards, where many but not all species can regenerate the tail, an important but not immediately vital organ (Arnold, '84; Maginnis, 2006). Why only some lizards can recover an entire tail whereas other caudate reptiles, with rare exceptions in crocodilians, are not capable of

regenerating organs? Also, why can lizards regenerate the tail but they cannot regenerate their limbs? (Fig. 1A). As mentioned in previous accounts (Alibardi, 2010a, 2014), lizards are amniotes and their regenerative ability has to deal with their immune system, a problem that anamniotes, like anuran tadpoles, larval, or adult urodeles do not have, in part explaining their outstanding regenerative capability (Mescher et al., 2013, 2016; Alibardi, 2017a). In amphibians, the regenerating limbs or the tail are attached to a body with many larval characteristics typical of neotenic vertebrates (Grigoryan, 2016). Therefore, the study of urodele models is very important for understanding the general aspects of development and regeneration, but gives relatively limited information on the specific problems hampering organ regeneration in amniotes. Because adult urodeles conserve many larval features, although they can reproduce offspring like the adults of the other vertebrates, the study of their regenerative abilities is similar to that of an advanced larva where the immune system is less active, similar to mammalian fetuses (Adzick and Longaker, '92). In anurans, the regenerative ability present in earlier tadpole stages ceases as the immune system becomes more effective approaching metamorphosis (King et al., 2001; Harty et al., 2003; Mescher et al., 2013; Godwin and Rosenthal, 2014; Alibardi, 2017a). This loss of regeneration capability makes anurans more interesting than urodeles in relation to the limitation of regeneration in amniotes, including mammals.

Anurans, like lizards, are therefore a very important vertebrate group for analysis of successful versus unsuccessful regeneration during different periods of their life cycle, such as when they become terrestrially adapted following metamorphosis (Mesher and Neff, 2006; Mescher et al., 2013, 2016). A recent study has shown that inflammation in anurans directly affects the formation of the apical epidermal cap (AEC), since the increases in the number of macrophages and lymphocytes in the blastema also invade the wound epithelium in stages approaching metamorphosis (Alibardi, 2017a). This process determines the loss of epidermal (stem) cells that in the embryo form the apical epidermal ridge (AER) or, in regenerating amphibians form an AEC, in which no regeneration occurs. It is known that during anuran metamorphosis macrophages are implicated in the destruction of numerous types of larval tissues (Fox, '77; Isutso et al., '93; Yoshizato, 2007). This occurs with the intervention of the endocrine system (mainly thyroid hormones and corticosteroids), and of the immune system, and may depend on the formation of adult lymphocytes that are no longer tolerant to embryonic or larval antigens present in the tail or in other organs of the tadpole since much earlier stages of development lead to their degeneration and replacement by "tolerated" adult tissue antigens. If this hypothesis is correct, the physiological process that leads the transition from tadpoles to terrestrial adults determines the limitation of tissue regeneration for terrestrial frogs, a hypothesis extendable to amniotes.

THE LIZARD MODEL OF TISSUE REGENERATION

Tail regeneration in lizards is a unique phenomenon among amniotes in which an embryonic-like organ, the blastema, remains connected to the body without immunological rejection (like fetuses in mammals). After tail or limb loss, the extravasating blood cells form a superficial clot in 3–6 hr postinjury, and underneath the clot the damaged tissues of the stump accumulate on their surface numerous free cells while the epidermis from the borders of the tail or limb gives rise to migrating keratinocytes that gradually insinuate underneath the clot over the following 4–7 days (Alibardi and Sala, '83; Alibardi and Toni, 2005; McLean and Vickaryous, 2011). The cells of the blastema originate from different tissues in both tail and limb that possess a variable number of 5BrdU long-retaining cells, putative stem cells, including blood cells from the bone marrow (Quattrini, '54; Simpson, '65; Cox, '69; Alibardi and Sala, '83; Alibardi, 2010a, 2014, 2015a, 2016a; Gilbert et al., 2015). The activation of c-myc and telomerase is among the initial proteins involved in the stimulation of formation of a blastema (Alibardi 2015b, d, 2016c). Also the presence of an upregulated gene coding for a MARCK-like protein in the transcriptome of the tail and limb of the lizard *Podarcis muralis* (Vitulo et al., 2017a), considered a specific initiator of limb regeneration in amphibians (Sugiura et al., 2016), suggests that also in lizard this protein is among the initial factors that trigger tail regeneration. Lactoferrin and *kfl4* (Kruppel-like factor 4) have been proposed among dedifferentiating proteins factors operating on tissues after tail amputation in lizard (Bae et al., 2014).

While in the tail numerous mesenchymal cells and relatively few hematogenous cells accumulate underneath the wound epidermis forming the blastema, in the limb numerous hematogenous cells and phagocytic granulocytes accumulate in the first 2 weeks postamputation. Macrophages with lymphocytes persist in the following 2–3 weeks, whereas mesenchymal cells remain scarce in the limb stump (Barber, '44; Alibardi, '86, 2010a,b, 2015a, 2016a,c Alibardi and Sala, '88). Lymphoblasts and the gamma globulin fraction increase in the blood of regenerating lizards after the third and fourth consequential tail amputations, when tail scarring becomes frequent (summarized in Alibardi, 2014). Immune cells and the gamma globulin fraction in the serum increases also in lizards kept in homeothermic conditions during tail regeneration that appears negatively affected (Alibardi, 2014). A high number of white blood cells are also present after extensive damage of the tail stump, such as multiple wounds or large wounds, or those produced after an oblique amputation of the tail (Baffoni, '50), or after hot cauterization (Alibardi, 2010a, 2013). In these cases, inflammation is stimulated and the tail retards regeneration and gives rise to a scar or a scarring outgrowth (Fig. 1B). Numerous granulocytes and macrophages are seen among the extensive damaged connective, muscle, nervous, and bone tissues of the limb, or in the

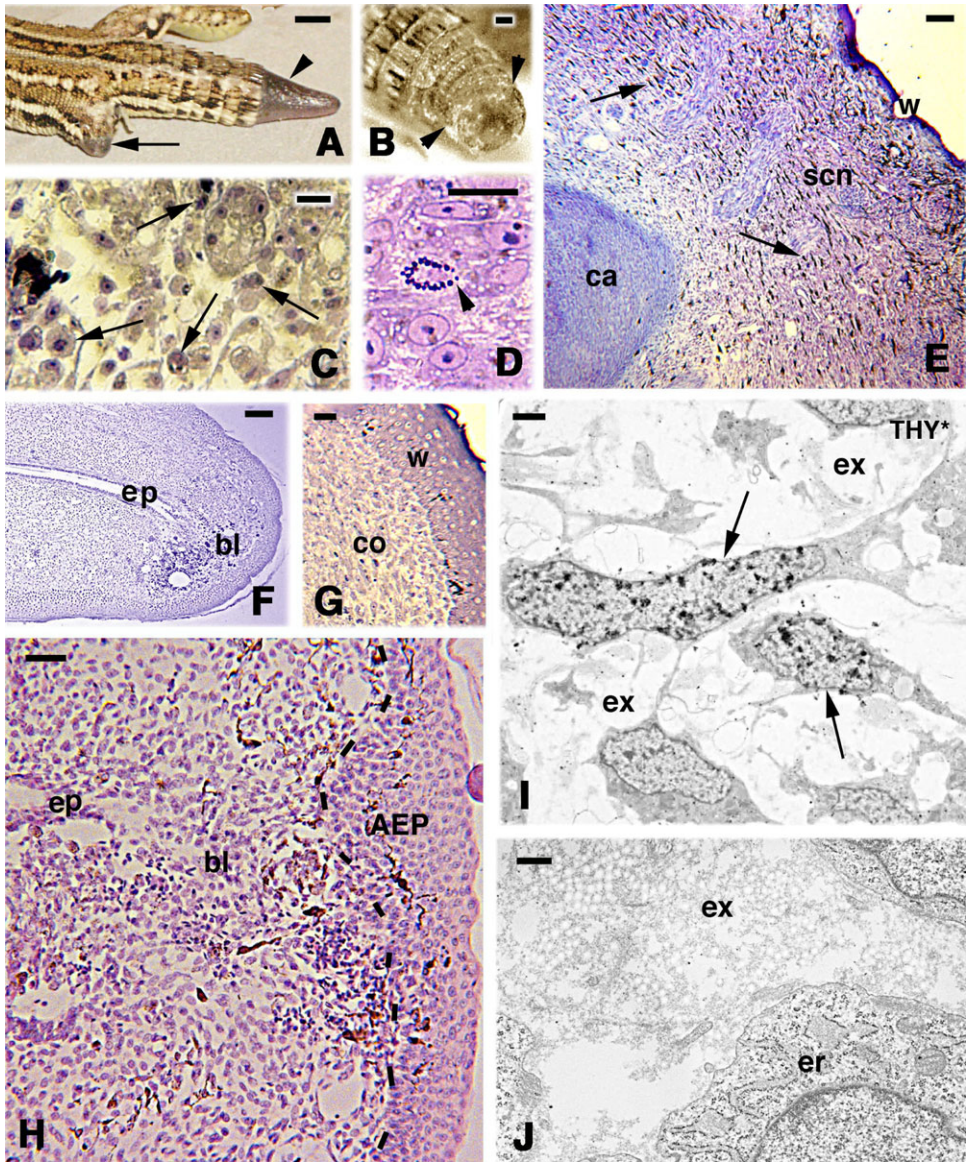

Figure 1. Macroscopic (A, B) and microscopic (C–J) aspects of tail and limb regeneration in lizards. (A) lizard (*Podarcis muralis*) with regenerating tail (arrowhead) and scarring limb (arrow) at about 18 days after amputation. Bar, 2 mm. (B) Scarring tail (arrowheads) after 45 days from amputation and cauterization. (*P. muralis*). Bar, 0.5 mm. (C) Macrophages (arrows) in the forming limb blastema at 12 days postamputation. Bar, 10 μm. *P. sicula*. (D) Dense mesenchyme of cauterized tail (scarring) at 15 days postamputation (the arrowhead points to a mastocyte; *P. sicula*). Bar, 10 μm. (E) Scarring tail blastema at 3 weeks postamputation and cauterization, showing the dense connective tissue rich in fusiform fibroblasts and chromatophores (arrows; *P. muralis*). Bar, 20 μm. (F) Regenerating tail after 16 days with regenerating ependyma that terminates in the apical blastema. (*P. sicula*). Bar, 50 μm. (G) Limb blastema at 18 days postamputation showing the connective tissue located underneath the apical and thick wound epidermis (*P. muralis*). Bar, 10 μm. (H) AEP (dashes) at the tip of the tail blastema at 15 days postamputation (*P. sicula*). Bar, 20 μm. (I) Tritiated thymidine labeled mesenchymal cells (arrows) in the blastema (*Anolis carolinensis*). Bar, 1 μm. (J) Ergastoplasmic-rich cells in a scarring tail blastema 15 days after cauterization, located within a reticulate-alveolate extracellular material made of collagen and amorphous matrix (*P. sicula*). Bar, 0.5 μm. Legends: bl, blastema; ca, regenerating axial cartilage; co, dense connective tissue; ep, ependyma (regenerating spinal cord); er, endoplasmic reticulum; ex, extracellular space/matrix; scn, scarring connective tissue. w, wound (regenerating) epidermis. [Color figure can be viewed at wileyonlinelibrary.com]

cauterized tail stump during the initial 2–4 weeks posttrauma (Alibardi, 2013; Fig. 1C and D).

Between 18 and 30 days postamputation, the initial mesenchymal blastema formed beneath the scab of the limb or of the cauterized tail is turned into a dense irregular connective tissue mass, made up of fibrocytes and a dense eosinophilic and Periodic Acid of Shiff (PAS) positive extracellular matrix, typical components of scar tissue (Barber, '44; Alibardi, 2010a,b; Fig. 1E). In contrast, a normally regenerating tail forms a mesenchymal blastema, a loose connective tissue located underneath a thick wound epidermis (Fig. 1F). The apical part of the wound epidermis of the tail, but not of the limb, gives rise to a more or less pronounced "apical epidermal peg" (AEP; Fig. 1G and H). This 6–12 cell layer thick epithelium possess a discontinuous basement membrane and is in contact with mesenchymal cells that also include cells originating from the apical ependymal ampulla (Alibardi and Sala, '88, '89), and that are innervated by apical nerves (Alibardi and Miolo, '90). The AEP develops neither a thick corneous layer nor a beta-layer, like in the epidermis of more proximal areas of the regenerating skin where new scales are formed. An AEP is not generally detected in the limb where the epidermis instead rapidly forms a continuous basement membrane and differentiates a corneous layer at 15–20 days postamputation. It is unknown whether the AEP corresponds to the AEC of amphibian blastemas. Like amphibians, the AEP is immune-reactive for p53/63, a protein that acts as a tumor suppressor on epithelial mersenchymal transformation (EMT; Alibardi, 2015c). This process is likely present during the initial phases of blastema formation in the tail of lizards but is rapidly blocked by the formation of the basement membrane in nonapical wound epidermis (Alibardi, 2012).

Autoradiography or immuno-labeling for cell proliferation markers indicates that numerous cells are dividing in the tail blastema (Simpson '61; Cox '69; Alibardi, 2010a; Delorme et al., 2012; Fig. 1I). In comparison to the tail, little proliferation instead occurs in the limb and in the cauterized tail blastema where a dense connective tissue is formed, suggesting limitation of cell movement and migration (Fig. 1J; Alibardi and Sala, '83; Ramachandran, '96; Nambiar et al., 2008; Alibardi, 2013, 2016a,c). The pattern of proliferation of the tail blastema only partially resembles that of fish and amphibians (Santos-Ruiz et al., 2002; McKusker et al., 2015), since the mesenchyme beneath the AEP has sparse proliferating cells, and the labeling index of this region is lower than in the apical regions of the regenerating spinal cord, cartilaginous tube, and in regenerating muscles (Cox, '69; Alibardi, '94; Fig. 2). Sparse cells incorporating tritiated thymidine or 5BrdU are present in the tail tip as it grows distally, whereas sparse labeled cells are detected in more proximal and differentiated muscles, cartilage, nerves, and spinal cord (Cox, '69; Alibardi, '94). The putative stem cells of the tail and limb, identified as cells retaining 5BrdU or tritiated thymidine for long periods (3–5 weeks or longer), have been shown to be present in numerous tissues of the stump, although with different frequencies (Alibardi, 2014, 2015a,b).

The intense proliferation of numerous tissues (Fig. 2) allows the growth of the tail, whereas in the limb a relatively lower proliferation occurs and a scar is formed at 3–4 weeks postamputation (Fig. 3A–E; Alibardi, 2013, 2016a,b). Pulse/chase experiments (6 days of 5BrdU injection in normal animal and then a chase period of 2 weeks) have shown that labeled cells are still present after 14 and 21 days of regeneration, although the labeling is diluted, in the tail blastema, wound epidermis, and in the regenerating scales (Fig. 3F). These experiments confirm previous histological and autoradiographic studies indicating that the cells of the blastema derived from the proliferation and movements of the cells from most of the tissues of the original tail. Numerous labeled cells also remain in the wound epidermis and in the AEP after 2 weeks of chase, but they have migrated from the basal layer to the intermediate and more external layers of the regenerating epidermis, supporting the process of epidermal expansion present in the regenerating tail (Alibardi, '94). In the limb, the labeling at 4 hr postinjection of 5BrdU is low in the repairing tissues, after 3–4 weeks postamputation, explaining the little or absent regeneration of the latter. However, after a pulse period of 6 days and a chase period of 2 weeks, the number of labeled cells progressively increases within the repairing injured tissues of the limb stump, indicating that in these areas a local proliferation has occurred but no distal cell migration has diluted the label (Fig. 3G). Cell proliferation is higher in the injured muscles and in the epiphyses of the long bones present in the stump of the amputated limb, including those not hit by the amputation (Alibardi, 2016a; Fig. 3G and H).

The ablation of the tail tip containing the AEP determines the temporary or permanent block of tail regeneration, and in case a new AEP is reformed the resulting tail is however shorter (Alibardi, 2010a, 2014). The formation of the AEP in addition to the ependyma of the regenerating spinal cord is also indicated by experiments of implantation of spinal cord in the tail to promote the formation of additional tails (Simpson, '61; Singer, '61; Whimster, '78; Alibardi et al., '88; Lozito and Tuan, 2016; Fig. 4A and B). The microscopic and ultrastructural analysis of the supernumerary tails produced in these experiments reveals the formation of an AEP in the growing front where the new tail is produced (Fig. 4C and D). The regenerating spinal cord, made up of a simple ependymal tube and few neural cells, elongates very closely near the AEP, likely contributing to the formation of the additional tail (Fig. 4C, and the inset). Also in this case, some nerves produced from the intrinsic neurons generated inside the implanted ependyma tube, grow until reaching the apical region of the additional tail (Fig. 4E; see details in Alibardi et al., '88). In summary, the presence of an AEP and of the apical front of the regenerating spinal cord, the ependyma ampulla, appears to be the two fundamental tissues driving tail regeneration in lizards.

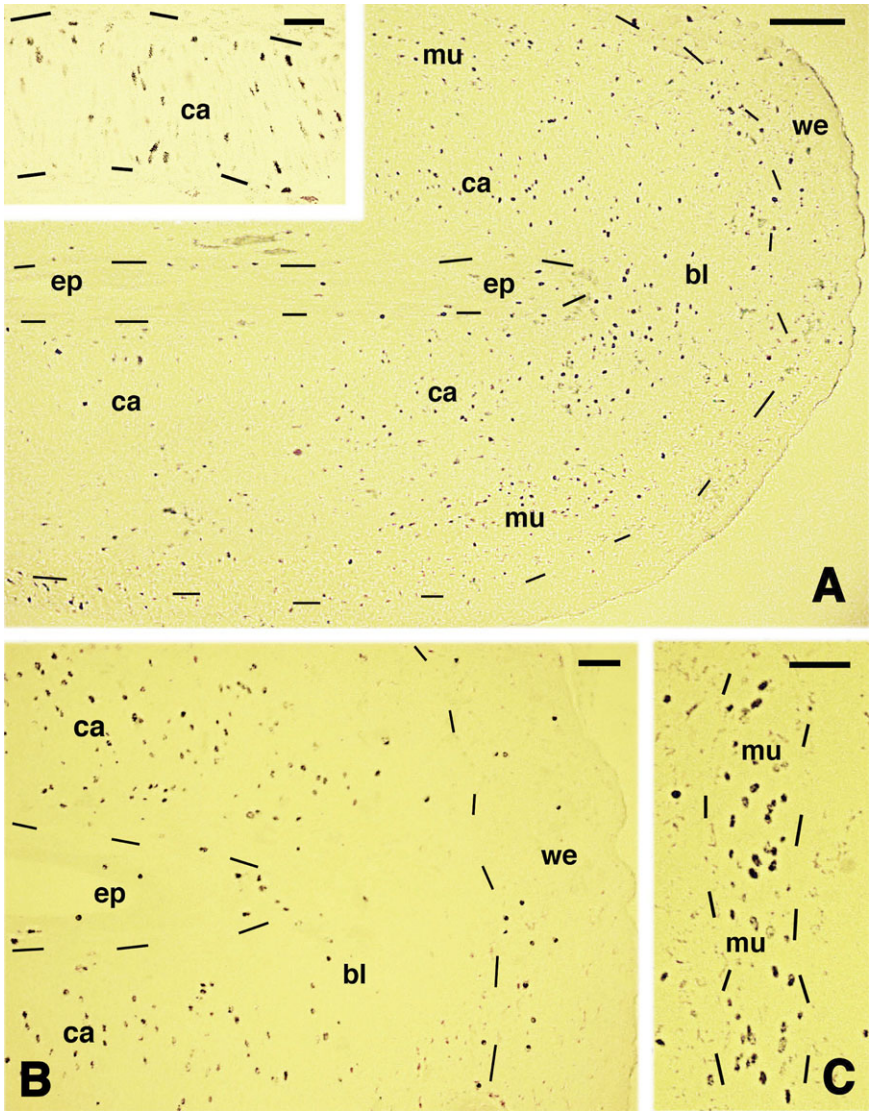

Figure 2. Tritiated thymidine autoradiography of unstained sections of regenerating lizard tail of about 3 mm (*Anolis carolinensis*), showing the localization of labeled (proliferating) cells as dark spots on the unstained tissues. (A) In a regenerating cone, the apical blastema contains sparse labeled cells that are more numerous in proximal regions where forming muscle and the cartilaginous tube are present around the central ependymal tube (indicated by dashes). Other dashes underline the wound epidermis. Bar, 50 μm. The inset (bar, 20 μm) shows a detail of the numerous labeled chondroblasts present in the regenerating cartilage (underlined by dashes). (B) Higher magnified view of the central apical region of the blastema in which few labeled cells are present in the mesenchyme and in the apical wound epidermis (surrounded by dashes). Labeled cells are more numerous in regions surrounding the ependymal ampulla (outlined by dashes), where procartilaginous cells are present. Bar, 20 μm. (C) Details of an apical pro-muscle aggregate (region where the new myomers are generated, surrounded by dashes) showing numerous labeled myoblasts. Bar, 20 μm. Legends: bl, blastema; ca, regenerating cartilage; ep, ependymal tube (regenerating spinal cord surrounded by dashes in the figures); mu, regenerating muscles; we, wound epidermis. [Color figure can be viewed at wileyonlinelibrary.com]

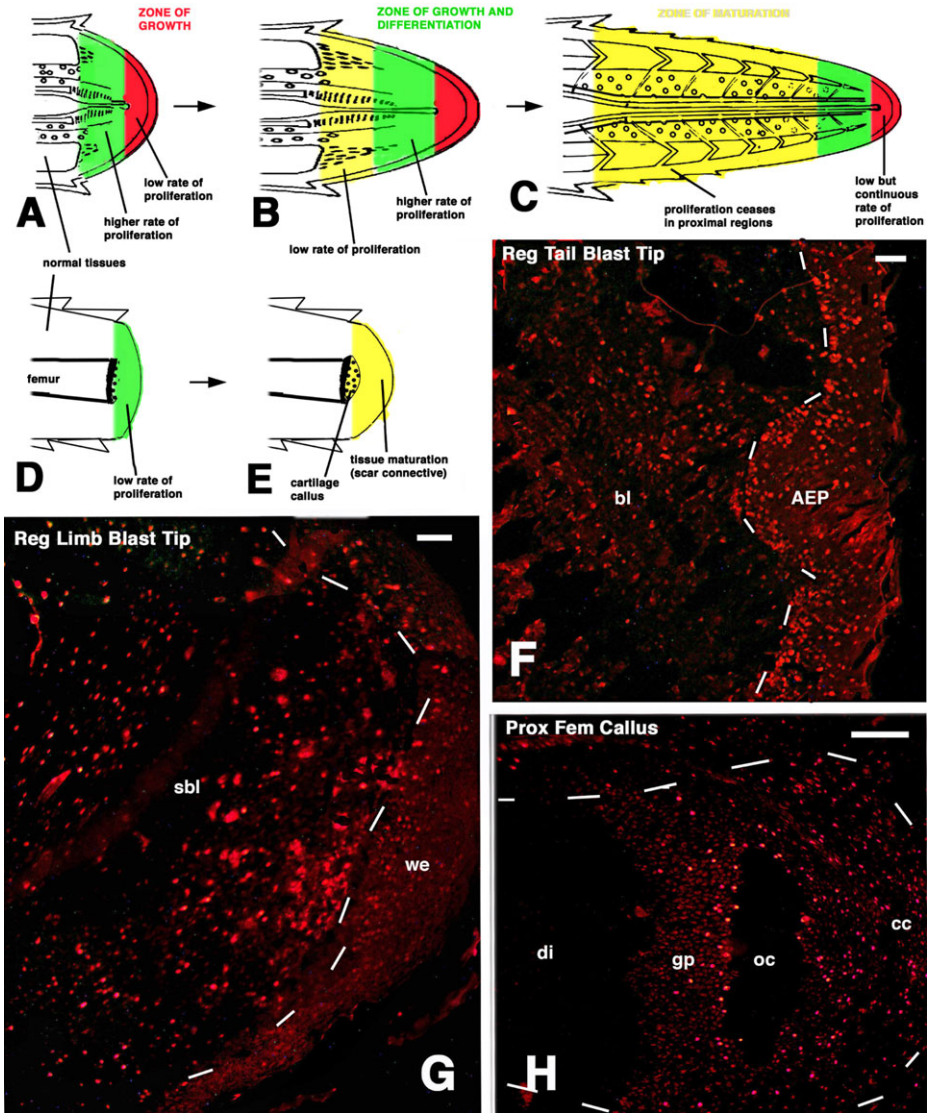

Figure 3. Schematic drawings illustrating the regions of growth and differentiation of the regenerating tail (A–C), limb (D–E), and the localization of proliferating cells (red with TRITC) after 6 days of pulse with 5BrdU and 2 weeks of chase (F–H, about 21 days of regeneration). (A) Regenerating blastema-cone subdivided into a very apical region (red) and a proximal region with muscle and cartilaginous cells at the beginning of differentiation (green). (B) Elongating cone, the more proximal region of which contains maturing tissues (muscle bundles with multinuclear myotubes, ependyma with tanicytes, cartilage made of hypertrophic chondrocytes, thick nerves, adipose cells accumulating fat, etc) with sparse 5BrdU and thymidine labeled cells. (C) Elongated and largely differentiated (extended yellow area) tail where proliferating cells are mainly detected in the subapical (green) and in the small apical region (red). (D) Initial limb blastema where some cell proliferation is present (green color). (E) Late scarring limb stump where the tissues have ceased their most proliferative stage and have healed the bone with a cartilaginous and ossified callus, while a dense connective tissue replaces the blastema (yellow color). (F) Apex of elongating tail cone, showing sparse labeled cells (red spots) in the blastema (bl), also distributed in the entire thickness of the apical wound epidermis including the AEP (underlined by dashes). Bar, 20 μm. (G) Apex of regenerating limb showing the accumulation of labeled cells in the scarring blastema (sbl) while few labeled cells are present in the apical wound epidermis (underlined by dashes). Bar, 20 μm. (H) Detail of the femur (di, diaphysis) located near the amputated limb surface (indicative position like in (E)), containing numerous labeled cells in the growth plate (gp), separated by an ossification center (oc) from the distal cartilaginous callus (cc) where numerous labeled cells are present. Bar, 50 μm. [Color figure can be viewed at wileyonlinelibrary.com]

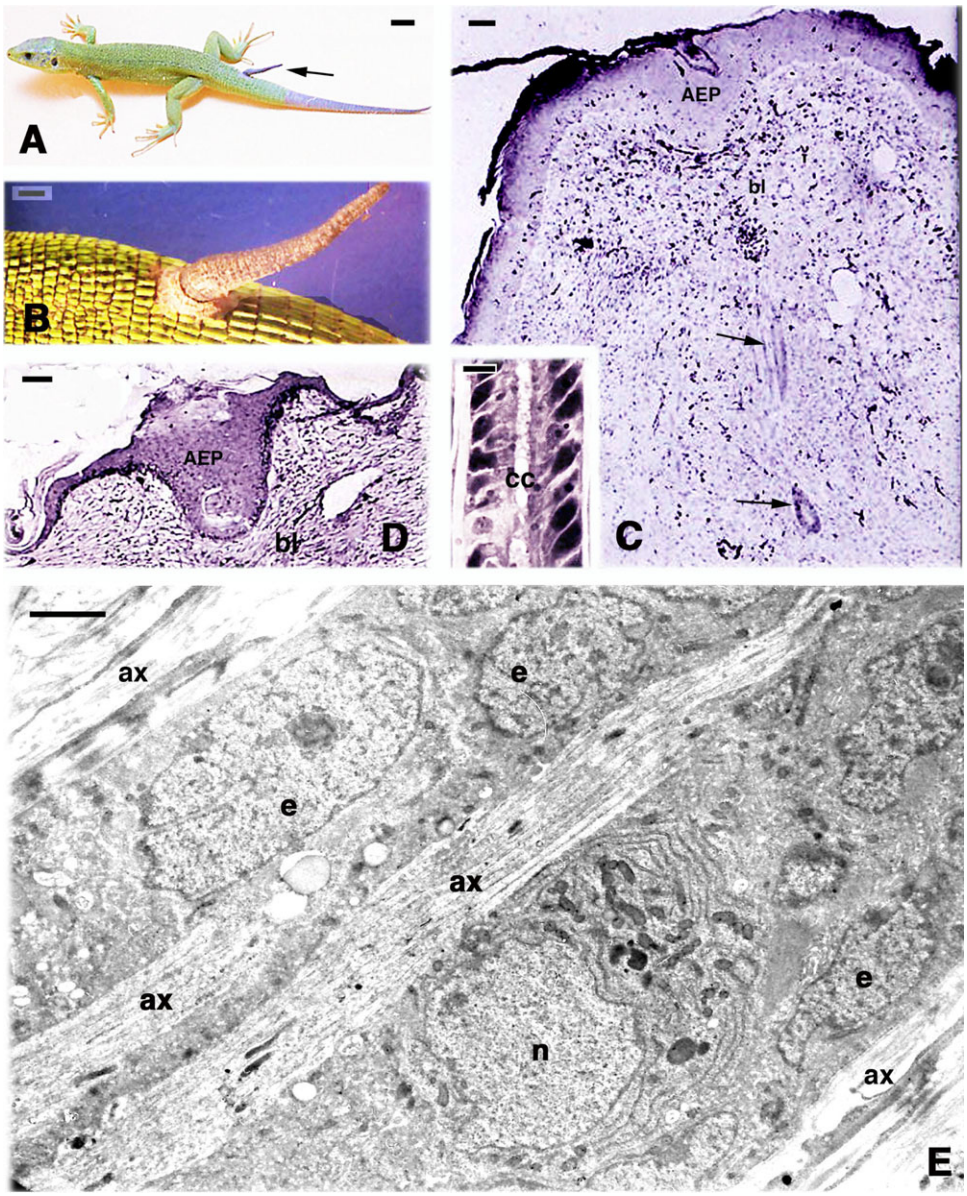

Figure 4. Induction of an additional tail (A, B) and microscopic images (C–E) after autoimplant of the caudal spinal cord collected from the amputated tail (details in Alibardi et al., '88). (A) Induced tail in *Lacerta viridis* at about 45 days from the implant. Bar, 1 cm. (B) Detail of additional tail covered by lines corresponding to scale formation. Bar, 1.5 mm. (C) Microscopic section of an additional regenerating blastema-cone at 22 days after the implant, showing the AEP, the blastema (bl), and the axial ependymal tube intercepted at two different levels (arrows). Bar, 20 μm. The inset (Bar is 10 μm) shows a higher magnification view of the ependymal epithelium and the central canal (cc). (D) Detail on the AEP of the blastema (bl) of a regenerating, additional tail. Bar, 50 μm. (E) Ultrastrucutural detail of the regenerating ependyma (indicatively corresponding to the ependyma indicated by arrows in (C)). This is composed of ependymal cells (e) among which thin axons (ax) are present and occasional differentiating neurons (n). Bar, 2 μm. [Color figure can be viewed at wileyonlinelibrary.com]

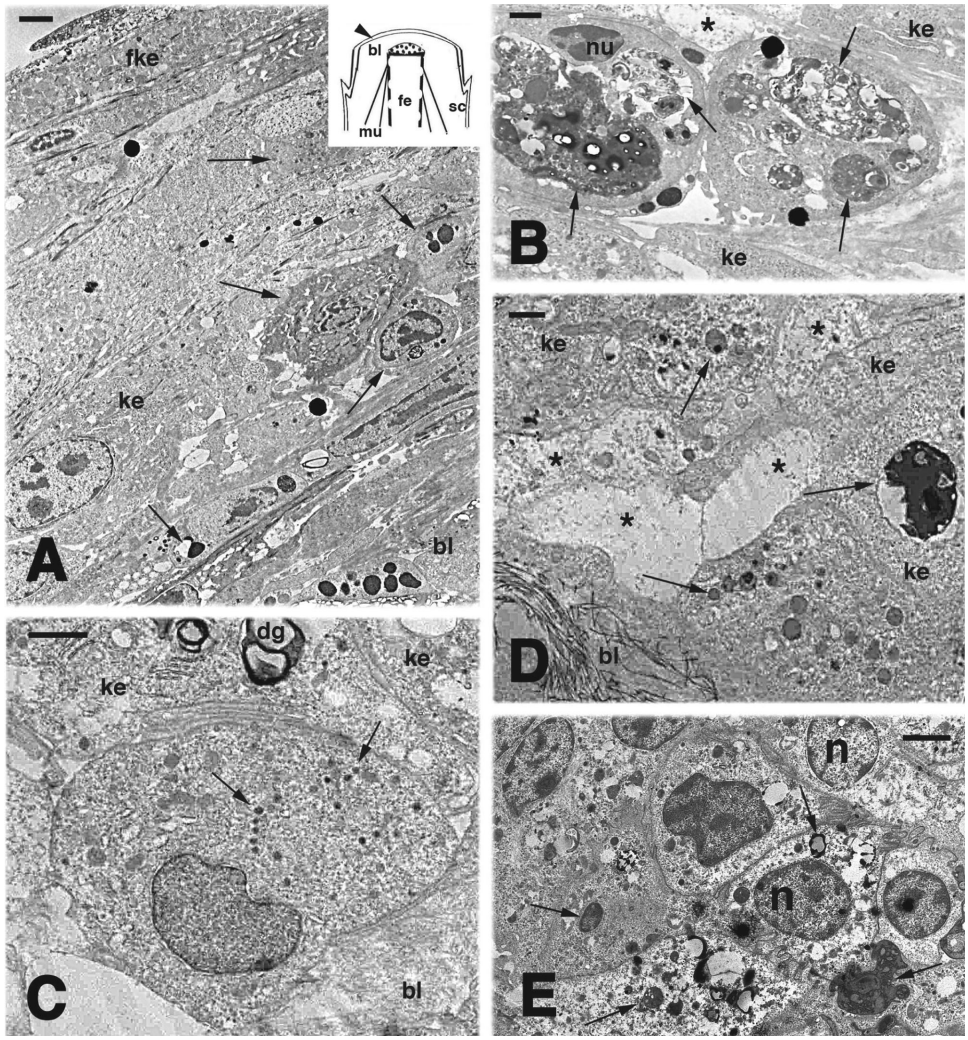

Figure 5. Ultrastructural aspects of the wound epidermis (A–D) and of the underlying blastema (E) 13 days after limb amputation in *P. muralis*. (A) Keratinocytes of the wound epidermis covering the stump (the drawing in the inset indicates the position of this area by an arrowhead). Flat and stretched keratinocytes are seen on the surface. Numerous phagocytes (arrows) are present among keratinocytes. Bar, 2 μm. (B) Detaild of two intra-epidermal phagocytes containing large phagosomes (arrows). The asterisk indicates a degenerated space within the epidermis. Bar, 1 μm. (C) Details of a likely phagocyte, possibly a neutrophil (arrows point to small granules), infiltrated among basal keratinocytes. Bar, 2 μm. (D) Numerous digestive organelles (arrows) are present in phagocytes located at the base of the wound epidermis. Asterisks indicate degenerated areas within the wound epidermis. Bar, 1 μm. (E) Group of phagocytes containing numerous phagosomes at various stages of digestion (arrows), located underneath the wound epidermis. Bar, 2 μm. Legends: bl, blastema; dg, degenerating body (secondary lysosome containing lipids); fke, flat external wound keratinocytes; ke, wound keratinocytes; nu, nucleus.

THE LIMB LACKS AN AEP LIKELY DUE TO AN IMMUNE PROCESS

The process noted for anurans where the AEC cannot form following the destructive action of macrophages and lymphocytes (Alibardi, 2017a), also occurs in the amputated limb of lizards, in which the numerous granulocytes in the first week and the persisting macrophages and lymphocytes in the two following weeks postamputation invade the blastema and colonize the epidermis determining the destruction of numerous bacteria but also of epidermal cells (Alibardi and Toni, 2005; Alibardi, 2010b, 2016a,c; Fig. 5). Impeding the formation of an AEP determines scarring in the limb. These observations suggest that the

evolution of a strong inflammatory and immune reaction in the amputated limb of lizards stops the reformation of a leading front (AEP), determining the failure of regeneration of the limb.

Many lizards possess a tail that has evolved autotomous planes along which tail loss is facilitated and followed by successful regeneration (Quattrini, '54; Bellairs and Bryant, '85; Fisher et al., 2012; Saangard et al., 2012). These anatomical planes of amputation allow losing the tail with minimum damage in many extant lizards, followed by little inflammation and immune cell colonization. The evolution of autotomous planes, absent in other reptiles that do not regenerate, including lizards devoid of autotomous planes, has been important for the evolution of regenerative abilities (see Alibardi, 2010a). Also tail amputation or a damage that does not directly affect autotomous planes (e.g., cutting the tail severing vertebrae outside autotomous planes or cut the regenerated tail where autotomous planes are absent) elicits a regenerative response. In the case of intervertebral amputation, regeneration is slower and initiates with the contribution of tissue in the autotomous planes. In the case of regeneration from a previous regenerated tail, the segmental intermuscle septa of the latter contain progenitor/stem cells. Another property that limits inflammation in the tail is the presence of potent antimicrobial peptides that are efficient inhibitors of bacterial growth and tissue invasion at the relatively low temperature present in their wounds, below 25°C (Alibardi, 2014). These antimicrobial peptides are likely very active at low temperatures, so that while bacteria cannot efficiently multiply at the relatively low temperatures they are efficiently killed by these Anti-Microbial Peptides (AMPs), a process that further limits microbial invasion and consequent inflammation.

Intensification of inflammation and of a likely immune response generally leads to scarring also in the tail (Alibardi, 2013, 2014). This is obtained by cauterization of the stump surface or of the spinal cord in the tail, or after sectioning obliquely the tail to produce an extensive damage and an uneven stump surface (Baffoni, '50). Another intervention that blocks or limits regeneration is the repetition of three to four tail amputations at the stage of tail elongation in a close succession (15–18 days apart), not allowing for a period of rest between successive amputations (Alibardi, 2010a, 2014). Also saline solutions containing Cd or Be can determine an initial delay of tail regeneration, as they also likely stimulate inflammation. Previous studies have shown that in these cases both lymphoblasts in the circulating blood and immunoglobulins in the serum (the gamma and beta-fractions) tend to increase after repetitive amputations, although no specific antibodies directed against mesenchymal antigens were detected (summarized in Alibardi, 2014). Therefore, the following discussion represents a hypothesis still to be confirmed or denied by specific studies. The experimental perturbation of the normal tail stump by massive tissue damage resembles the conditions present in the transected limb and elicits an intense inflammatory reaction. These studies have suggested that while mesenchymal and epithelial cells exposing embryonic antigens to the immune cells of the blastema, are somehow tolerated during the first regeneration, this does not occur in the tail under intensified inflammation. In the latter case, it is hypothesized that immune cells attach to blastema cells by recognizing the latter as nonself and activate an immunological reaction as in the limb. This hypothesis however needs further studies to be confirmed.

In conclusion, current information indicates that a low inflammation allows the formation of an AEP in the tail, and this driving microregion is somehow capable of coping with the immune surveillance, leading to tail regeneration. This is apparently obtained by establishing in the blastema a proliferating center while the more proximal tissues can express their self-autonomous growth potential (Fig. 3A–C). The new muscles, nerves, and axial cartilaginous tube and spinal cord are recognized as self by the immune system, and they are likely stimulated by different growth factors.

GROWTH FACTORS STIMULATE TAIL AND LIMB REGENERATION IN LIZARD

Among growth factors, in particular FGF1, FGF2, and their receptors stimulate tissue regeneration in amphibians (Cannata et al., 2001; Dungan et al., 2002) and lizards (Alibardi, unpublished observations). FGF2 and FGF1 are localized in the regenerating lizard tail, especially in the wound epidermis and in the regenerating spinal cord where they are present in axons and in the few neurons present (Alibardi and Lovicu, 2010). In the limb, FGF1 and FGF2 are detected in the wound epidermis that covers the stump at 2 weeks postamputation but the immunoreactivity rapidly disappears as the limb turns into a scar, and the epidermis differentiates a corneous layer in the following 2 weeks (Alibardi, 2012). Other studies have indicated that the apical ependymal ampulla and the AEP contain FGF8, whereas FGF10 is more localized in the mesenchyme of the blastema (Alibardi, 2015c, 2016; Fig. 6A–D). FGFs appear particularly immunolocalized along the incomplete basement membrane of the apical wound epidermis and of the AEP, as is indicated by ultrastructural immunogold labeling for FGF2 (Alibardi, 2012; Fig. 6E). These observations, considered together with the localization of p53/63 in the AEP (Alibardi, 2015c), strongly indicate that the AEP of lizards has at least some markers of the AEC in amphibians (Dungan et al., 2002; Christensen et al., 2001; Giampaoli et al., 2003; Yun et al., 2013) and of the AER of vertebrate embryos (Martin, 1998).

The importance of FGFs as neurotrophic factors is also seen following the injection of FGF1 or FGF2 inhibitors into the tails, an intervention that delays or even stops tail regeneration (Pillai et al. 2013; Narayanan, 2015). By contrast, the administration of FGF1 and FGF2 induces the regrow of a regenerating blastema not only in the tail but also in the amputated limb within 30–70 days in the lizard *P. muralis* (Alibardi, unpublished

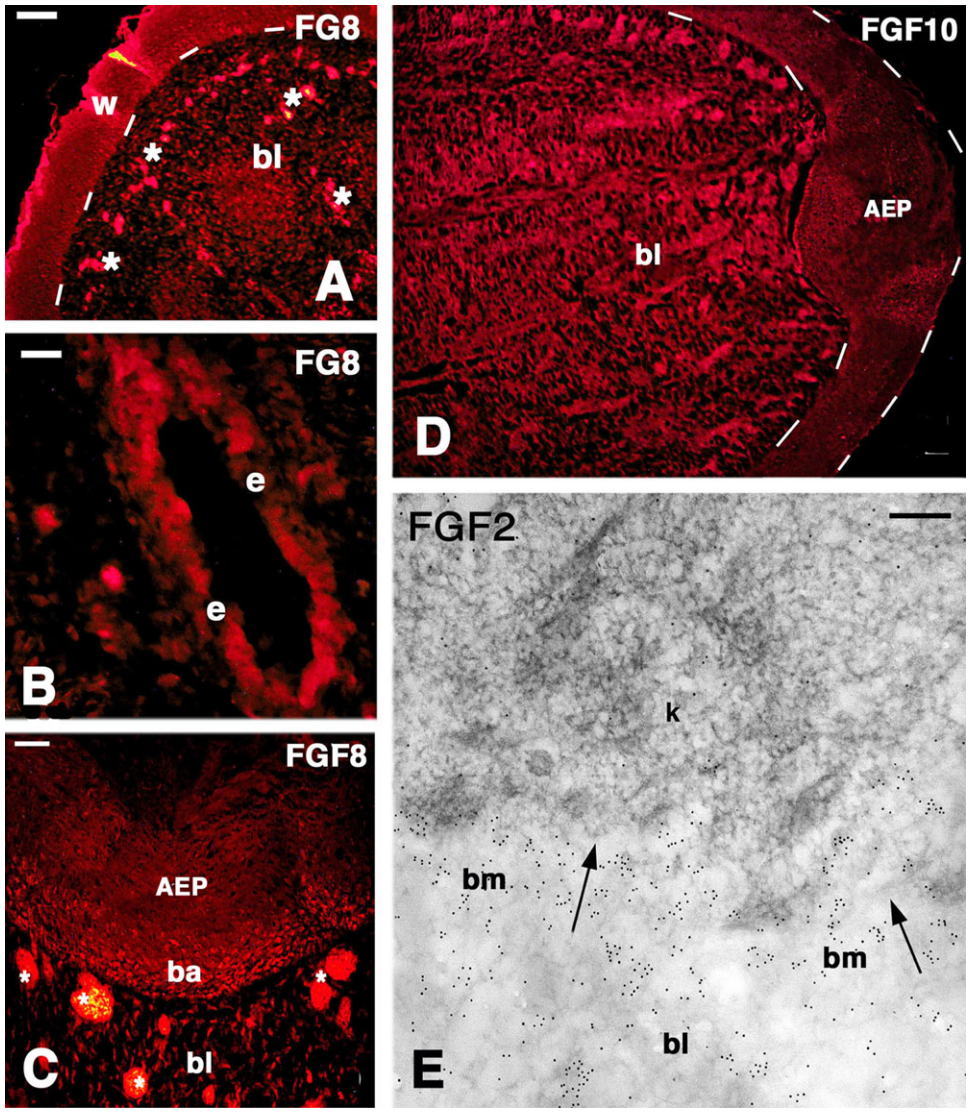

Figure 6. TRITC immunofluorescence localization of FGF8 (A–C), FGF10 (D), and FGF2 (E), in lizard blastema (*Podarcis muralis*). (A) General view of the blastema covered by a thick wound epidermis (w, underlined by dashes). Asterisks indicate sparse autofluorescent blood vessels. Bar, 20 μm. (B) Immunostained ependymal ampulla (e) present near the tail tip. Bar, 10 μm. (C) Details of AEP with labeled cells in the basal layers (ba). In the underlying blastema (bl) autofluorescent blood vessels are present (asterisks). Bar, 20 μm. (D) Blastema immunostained for FGF10 showing immunofluorescent apical blastema but little in the wound epidermis and AEP (outlined by dashes). Bar, 10 μm. (E) Ultrastructural details of the boundary region (arrows) between a basal keratinocyte (k) of the apical wound epidermis and the underlying blastema (bl) after immunogold staining for FGF2. Most of the gold labeling is present along the discontinuous basement membrane (bm). Bar, 200 nm. [Color figure can be viewed at wileyonlinelibrary.com]

observations). This experiment produces 2–4 mm long cone-shaped or flat outgrowths during this period (Fig. 7A–C). Histological studies have indicated that a thickening of the wound epidermis or a true AEP is formed at the tips of these outgrowths, which contain an immature axial cartilage rod (Fig, 7D), often subdivided into two parts recognized as cartilaginous tibia and fibula (Alibardi, unpublished observations). Large and peripheral areas, outlining the regenerated tibia–fibula, are composed of immature cartilage, and only the more central region forms hypertrophic chondrocytes that result metachromatic at

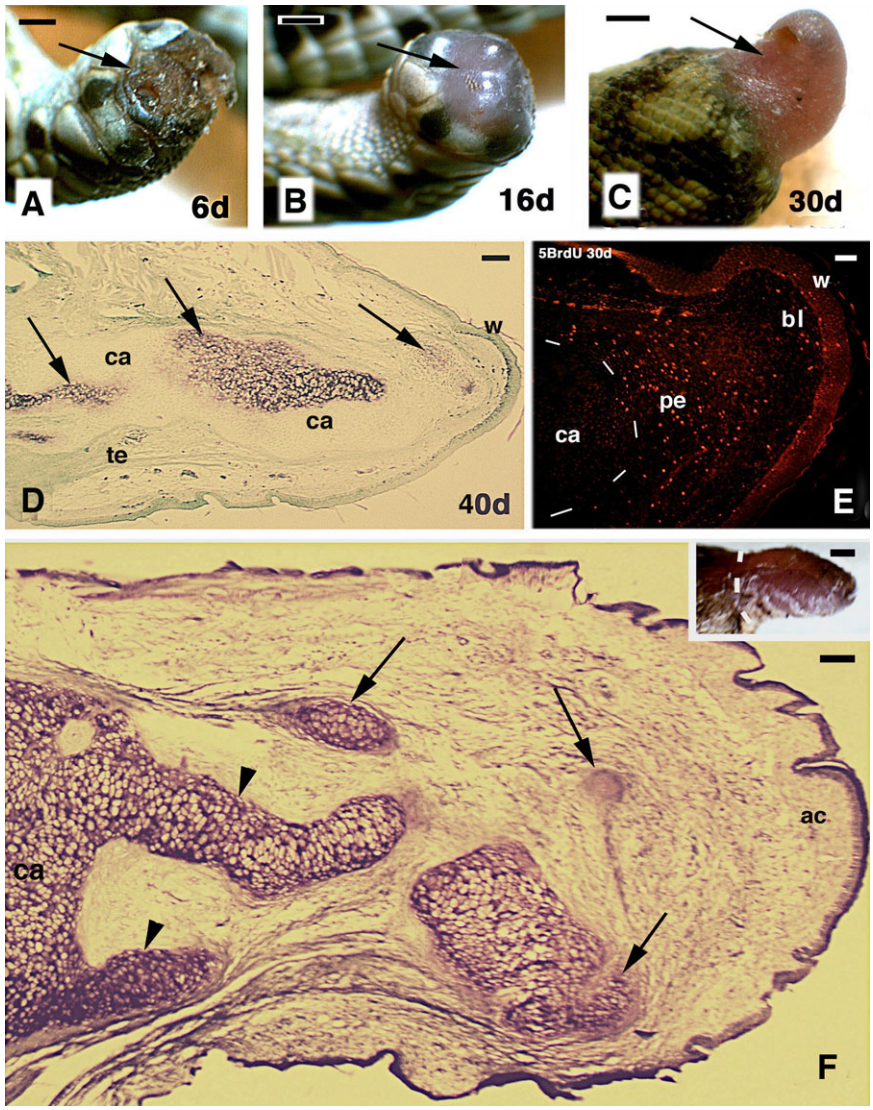

Figure 7. Macroscopic effects of FGF1 injections on amputated hindlimbs (A–C), and their microscopic structure (D–F) in *P. muralis*. (A) Aspect of amputated limb stump (arrow) after 6 days. Bar, 1 mm, (B) Aspect of the limb covered by a healing blastema (arrow) 16 days after amputation. Bar, 1 mm. (C) Aspect of regenerated limb outgrowth at 30 days postamputation. Bar, 1 mm. (D) Longitudinal section of limb outgrowth stimulated by FGF1, fixed at 40 days postamputation. An axial, still largely immature cartilage (ca, arrows indicate the inner metachromatic cartilage) occupies the entire length of the outgrowth up to the apical wound epidermis (w). A tendon-like connective belt (te) connects the cartilage to more proximal tissues in the stump. Bar, 50 μm. (E) Outgrowth of 40 days, showing 5BrdU labeled cells located at the apical "blastematic" tip (bl), in the wound epidermis (w), and in the distal perichondrium (pe) of the cartilaginous rod (outlined by dashes). Bar, 20 μm. (F) Regenerated limb at 40 day postamputation (visible in the inset, Bar, 0.5 mm), after FGF2 and retinoic acid treatment. The distal cartilaginous rod (ca) is subdivided into two rods of cartilages (arrowheads), and other nodules are seen more distally (arrows), near the apical connective tissue at the tip of the outgrowth (ac). Bar, 50 μm. [Color figure can be viewed at wileyonlinelibrary.com]

30–40 days of limb regeneration. Most of the regenerated cartilage becomes mature at 50–60 days of regenerations. Almost no muscle bundles are regenerated in these outgrowths that are instead composed of a dense and irregular connective tissue and tendon-like cords contacting the axial cartilage and the old muscles in the stump. Proliferating cells, detected after injection of 5BrdU, are few and sparse in mature regions of the outgrowths at 40–60 days of regeneration, whereas most of the labeled cells are present in the apical region of the outgrowths, in the wound epidermis, and in the cone-shaped apical perichondrium (Fig. 7E). This indicates that the outgrowths are continuously growing during the studied periods (40–70 days of regeneration). Cell proliferation however is reduced at 70 days postamputation, but it is however likely that the outgrowths slowly continue to elongate with the somatic growth of the lizard. It is likely that somatic growth in addition to regeneration can explain the length, over 10 or even 20 mm, detected in rare cases of tail-shaped regenerated limbs in lizards (Marcucci, '25, '30).

The addition of agar bits soaked with all-trans retinoic acid in a posterior-lateral region of the early limb outgrowths determines the branching of the cartilaginous condensation in its distal-most region after 40–60 days of limb regeneration, suggesting the formation of apical cartilaginous segments that may represent rudimentary autopodial elements (Fig. 7F). A further study using more precise site injections of retinoic acid could provide further information. Although the experiments so far conducted have not induced the regeneration of an autopodium (foot or hand), this may be attempted in the future with more precisely localized injections of specific signaling proteins known to determine digit formation (Sanz-Ezquerro and Tickle, 2001). In fact, as the molecular information on signaling proteins in the regenerating tail and limb will become available (Vitulo et al., 2017a,b; see later), the selection of other signaling proteins that could be utilized for stimulating the formation of the autopodium in the limb will be "at hand." Using this information on the specific and more upregulated genes expressed in the tail AEP, further attempts to induce the formation of a functional AEP and of skeletal elements of the autopodium also in the amputated limbs of lizards can be attempted.

TGFβ1 expression in the regenerating blastema-cone stages is present in low amount in the blastema, whereas activin and *SMAD2* are upregulated in relation to the increased cell proliferation of the blastema (Gilbert et al., 2013). Also *snail2*, a gene marker of EMT is significantly upregulated in the tail blastema, confirming the presence of an initial EMT in lizard wound epidermis (Alibardi, 2012). Finally, it is reported that, while Nerve Growth Factor (NGF) stimulates tail regeneration, the treatment with TGFβ and EGF retards or inhibits tail regeneration as the former stimulates precocious chondrogenesis whereas the latter accelerates epidermal differentiation and collagen deposition (Kurup and Ramachandran, 2011). The above studies suggest that genes coding for growth factors and other signaling proteins are responsible for triggering tail regeneration.

GENES UPREGULATED IN THE TAIL AND LIMB EXPLAIN REGENERATION VERSUS SCARRING

To understand the mechanism determining tail regeneration in lizard, recent molecular analysis on the transcriptome of the regenerating tails in the lizards *Anolis carolinensis* and *Gekko japonicus* indicates that numerous genes involved in signaling pathways, metabolic, wound response, immunity, developmental processes, and myogenesis are activated (Hutchins et al., 2014, 2016; Liu et al., 2015). These studies have identified hundreds of up- and downregulated genes during the regeneration of the tail that appear typical of wounding, inflammation, hormonal, and differentiating processes. Despite these pioneer studies, the key genes activating the process of regeneration in lizards remained undetected. In fact, only mutations affecting tail regeneration, presently unknown might give clues to the roles of specific genes on lizard regeneration. Another possibility in the future would be the experimental alteration of gene expression by gene knockout of specific lizard genes to determine whether and how they affect tail regeneration. This intervention should however initially provide some clues about the genes important to be silenced to identify the key or master genes that are essential to orchestrate a macroscopic process such as the regeneration of the tail.

To identify the key genes determining tail regeneration in lizards, we have recently utilized a different approach, namely the comparative evaluation of gene expression in tail versus limb during regeneration in the same animals (Vitulo et al., 2017a,b). Although the cells in the limb have the same genes present in those of the tail, the potential regenerative program appears to be halted in the limb, due to the amount of tissue destruction that is mobilizing leucocytes and macrophages, cells that invade the limb stump and activate high proteolytic and oxidative metabolism, two processes favoring scarring. The comparison between the transcriptomes of the regenerating tail and limb has permitted us to determine the main differences in gene expression between a regenerating organ versus a nonregenerating organ within the body of the same individuals (Figs. 1A and 8). This has allowed, for the first time, the identification of the key genes determining regeneration in the tail and scarring in the limb. Among the hundreds of genes upregulated in the tail and limb, in particular those exclusively overexpressed in the two organs, they are classified into three main categories, on which the following hypotheses are presented: (1) noncoding RNAs and coding genes for signaling proteins, which are the key genes stimulating regeneration; (2) Inflammatory-Immune genes, which contrast or allow the key genes to be expressed; (3) cellular and extracellular functional genes that represent physiological genes directly or indirectly activated as a consequence of the activation of (1) and (2) and include

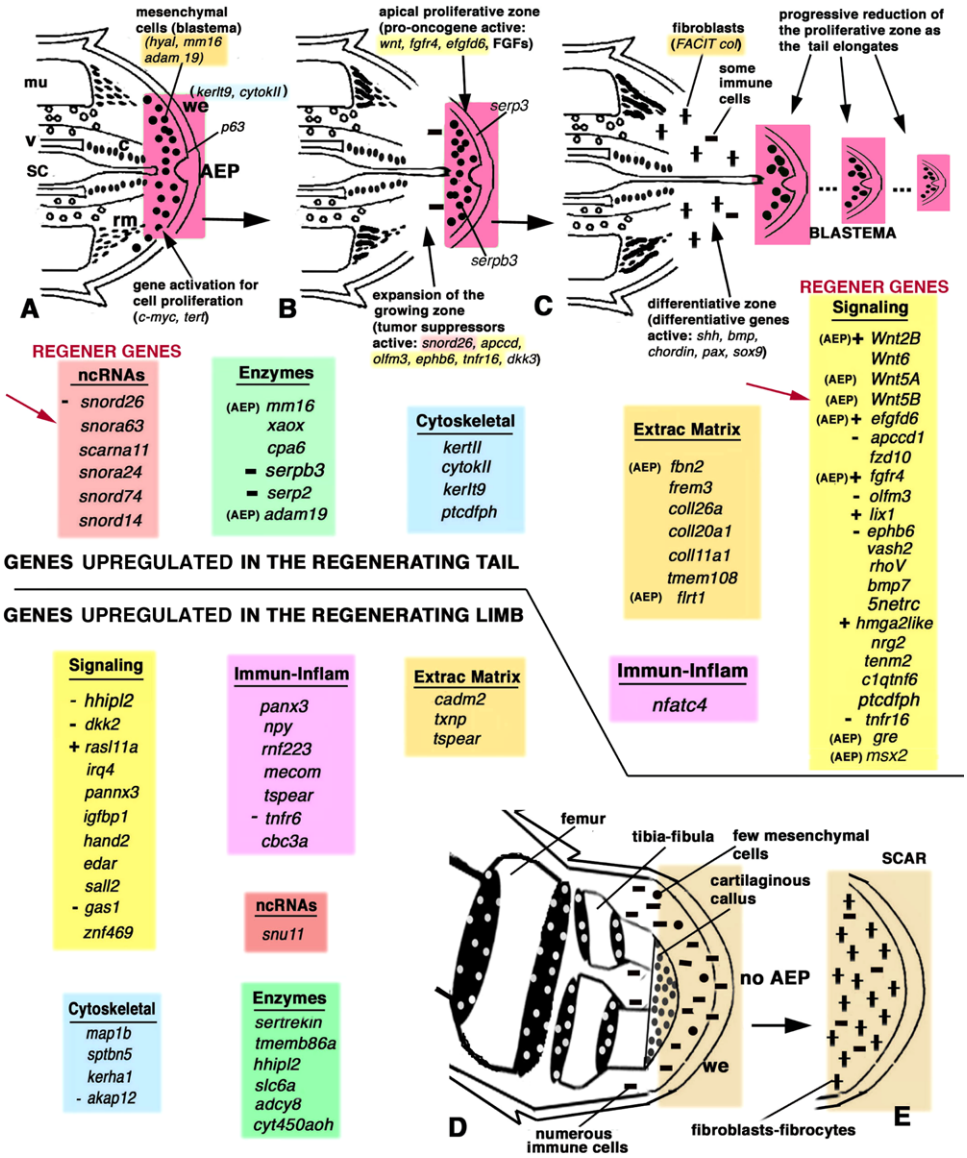

Figure 8. Schematic drawings showing an hypothesis of how some of the main upregulated genes, subdivided in differently colored categories, and implicated in the regeneration of the tail (A–C) and the limb (D and E) can drive regeneration in the tail and scarring in the limb. (A) shows the coniform blastema, with the apical part (red), dominated by the Wnt-signaling pathway that keeps a continuous proliferation of this region as it becomes smaller and smaller while the tail elongates in B and C). Signaling proteins stimulating proliferation are indicated with a +, whereas inhibitory proteins are indicated with a –. The red arrows indicate key genes for regeneration. (B) shows that the proliferating blastema (red) has moved forward, leaving behind the differentiating region, possibly dominated by regulative proteins (–) for cell proliferation. Immune cells (macrophages and lymphocytes) are sparse or not active in the tail blastema. (C) Progressive reduction of the blastema (red) in elongated tails. (D) Limb blastema dominated by Wnt inhibitors (indicated with a –) and occupied by numerous immune cells (macrophages and lymphocytes). (E) Scarred blastema filled with fibrocytes and sparse immune cells (macrophages). Legends: AEP, apical epidermal peg; c, regenerated cartilaginous tube; mu, muscles; rm, regenerating muscles; sc, spinal cord; v, vertebra; we, wound (regenerating) epidermis. Most of the gene names, in *italicus,* follow the nomenclature (see details in Vitulo et al., 2017a). [Color figure can be viewed at wileyonlinelibrary.com]

cytoskeletal, extracellular matrix, and metabolism enzymes (Vitulo et al., 2017a; Fig. 8).

Genes for Key Signaling Proteins, Immunity, and Cell-Extracellular Function

The transcriptome study on the coding genes indicated a main upregulation of the Wnt-signaling pathways in the tail blastema, a signaling circuit that is absent in the scarring limb (Fig. 8). The main Wnt genes (*wnt 2b*, wnt *5a, wnt5b, wnt6*), together the *edgfd6*, *fgfr4*, *grem,* and *msx2* are known to be expressed by an active AEC and AER and their connected mesenchyme (Kawakami et al., 2006; Lu et al., 2008). These genes are stimulating cell proliferation in the growing front located at the tip of the regenerating tail (Alibardi, 2017b; red area in Fig. 8A–C). In contrast, in the limb-signaling genes such as *dkk2*, *hhipl2*, *gas1,* etc. (see Fig. 8D and E) inhibit the Wnt pathway and do not allow the formation of a proliferative front but instead stimulate cell differentiation, tissue repairing, and eventually scarring (Vitulo et al., 2017a). Few Inflammatory-Immune genes are upregulated in the tail (*nfatc4*), whereas important inflammatory-immune genes (*panx3*, *npy*, *tnft6*, *mecon*, *tspear*, etc.; see Fig. 8) are more numerous and highly activated in the limb before and during scarring. High upregulation of serpin genes (*serpb3* and *serp2*) only in the regenerating tail, proteins that are involved in limiting inflammation and the activity of macrophages and lymphocytes, also suggests induced immune tolerance to blastema cells.

Only a general interpretation on the overexpression of some enzymes in the tail and limb can be presently offered (Vitulo et al., 2017a). The high upregulation of xantine-oxidase-like enzyme (*raox*) may indicate a high metabolism of nucleotide bases in the tail blastema for DNA and RNA metabolism, like in some tumors. The high expression of carboxypeptidase-6 (*cpa6*) may somehow be involved in the metabolism of amino acids and neuropeptides. The high upregulation of serpin genes (*serpb3* and *serp2*), coding for two serine-protease inhibitors, suggests a strong control of intracellular protein degradation in the tail blastema, in favor of protein biosynthesis. Recent studies, however, have indicated that serpins also intervene in limiting the action of degrading enzymes secreted in macrophages and T-lymphocytes, therefore lowering the destructive action of immune cells on cancer cells, a process indicated as immunoevasion (Ashton-Rickardt, 2015). In the limb, many enzyme and carrier protein genes are overexpressed (*sertrekin*, *scl6a, hhip12*, etc.; Fig. 8) and appear in relation to the intense catabolic and remodeling processes present in the blastema of the limb in the stages analyzed, 16–18 days postamputation (Alibardi, 2010b, 2014, 2016a: Vitulo et al., 2017a).

KEY GENES FOR NONCODING SMALL RNAs

Among the category of genes essential to stimulate regeneration, the noncoding and exclusive upregulated RNAs found in the tail blastema but absent in the limb, belong to snoRNAs (small nucleolar RNAs; Fig. 9). Although the total number of small RNAs detected in our transcriptome study on *P. muralis* likely represents a fraction of the real number of the noncoding RNAs activated in the regenerating tissues, most of the snoRNAs (35) and miRNAs (5) are common between the regenerating tail and limb, but it is the degree of their expression that varies between the two organs (Fig. 9). SnoRNAs intervene in the methylation and pseudouridynation of rRNAs that influence the regulative activities of ribosomes in transcription, both in terms of speed, precision, length of translated mRNAs, and timing-temporal frame of translation; the latter process is of particular importance during development to assign cells to specific differentiation fates (Dieci et al., 2009). The control of snoRNAs during processes of cellular proliferation is important as the upregularion of ribosome synthesis is often linked to neoplastic degeneration (Derenzini et al., 2017). Another function of snoRNAs is related to their activity during the condensation of the chromatin, maintaining open some regions of the chromosomes for transcription, and controlling also chromatin remodeling (Dieci et al., 2009).

Most of the detected overexpressed snoRNAs (23) are more upregulated in the tail than in the limb while few are more intensely expressed in the limb, basically *snord 98* that shows the highest expression (Fig. 9A–D). Ten snoRNA are much more regulated in the tail blastema than in the limb blastema, and one (*snosnr60z15*) has over 250-fold expression in the limb blastema (Fig. 9D). Eleven snoRNA genes are however exclusively expressed in the regenerating tail blastema (Fig. 9E), whereas only one snoRNA is exclusive to the scarring limb, another indication that these short RNAs are part of the "regenerative genes" necessary to originate a new tail. However, given the scarce information on the biological role of specific snoRNAs on morphogenetic process, there are few clues on their specific role for lizard regeneration that can be given at the present time. The snoRNAs more highly expressed in the tail in comparison to the limb include *Snord 2, 17, 22, 49, U3,* and *snora 81*, and the most highly expressed gene in the tail, *snosnr60Z15*, is likely involved in the regulation of biosynthetic activities that maintain the proliferation and growth of the tail blastema. The highest expresses limb gene, *snord 98*, over 210 folds of expression, may instead be related to the intense inflammation and fibroplasy that takes place in the limb, but specific information are missing for all the above snoRNAs.

Among the most highly expressed, exclusive snoRNAs of the tail blastema, *snord26* is a tumor suppressor and this RNA may act as a negative regulator of cell proliferation in the apical front of the blastema (indicated with a in Fig. 8). Another overexpressed type of RNA, *scarna11*, is present in Cajal bodies that are nuclear organelles involved in the formation of the splicing complexes and of the elaboration of the telomerase enzyme, a RNA–protein complex controlling cell proliferation. Telomerase localization has been found in sparse cells of the lizard blastema,

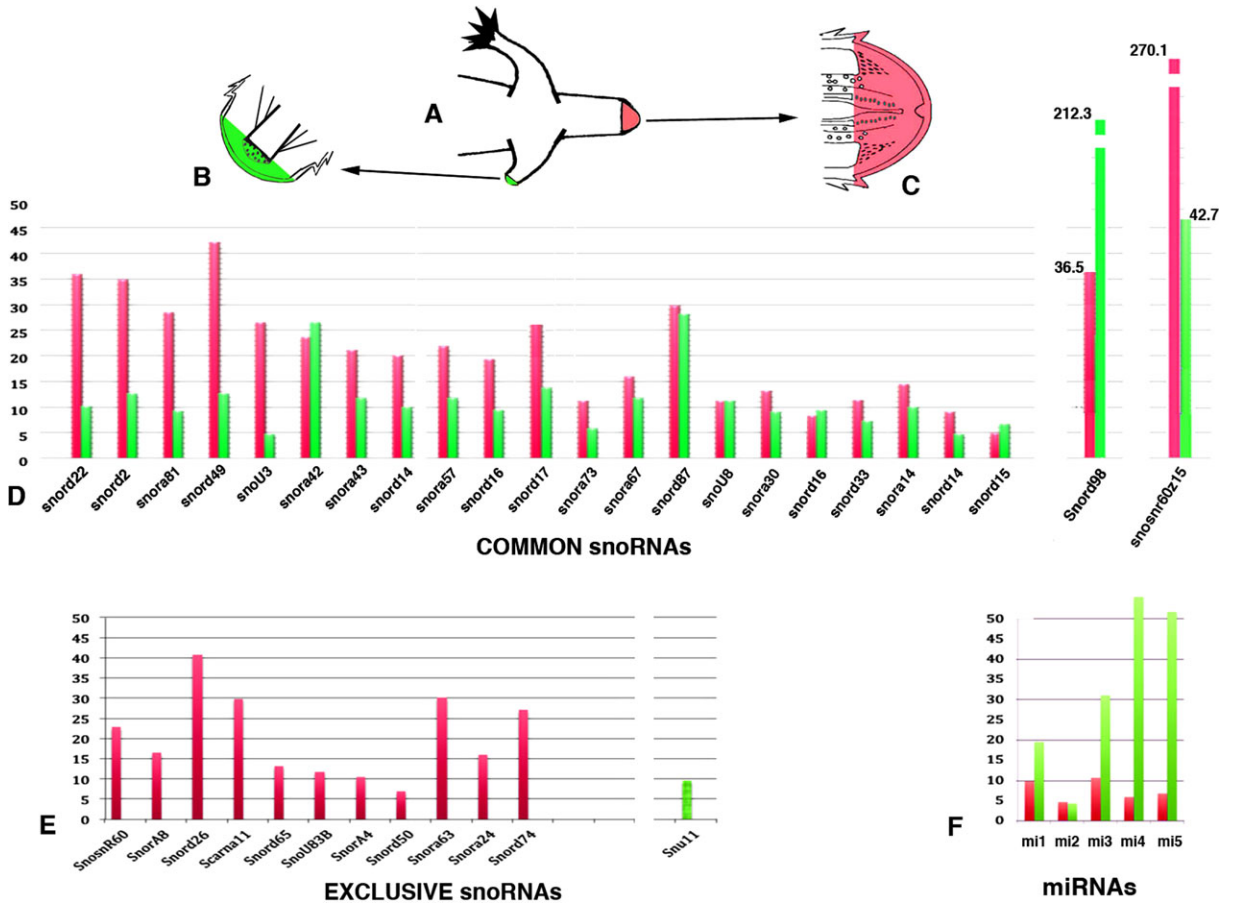

Figure 9. Schematic representation of a lizard trunk (A) with regenerating limb (B, green) and tail (C, red), referred to *P. muralis*. The histograms show the folds variations (numbers in ordinate) of the snoRNAs (D and E) and miRNAs (F) upregulated in the regenerating tail (red) and limb (green) transcriptomes. (D) indicates the common snoRNAs present in the limb (green) and tail (red) blastemas. (E) indicates the upregulated folds of snoRNAs exclusive of the tail blastema (red) and of the limb blastema (green). (F) reports the folds variation of miRNAs in the regenerating tail blastema (red) versus the regenerating limb blastema (green) (see the text for explanation). [Color figure can be viewed at wileyonlinelibrary.com]

suggesting it is involved in their numerous cycles of proliferation that must be however regulated after cells exit the apical blastema front (Alibardi, 2015b). This passage from the apical to more proximal regions of the regenerated tail is roughly indicated in Figure 3A–C by the red to green regions or from the red to white regions in Figure 8A–C. The biological role of the other upregulated *snord 60* and *snord 74* is unknown, but they likely are also stimulating cell proliferation and blastema growth. The unique limb-expressed *snu11* is involved in chondrogenesis and osteogenesis, two differentiating processes active in the scarring limb (Vitulo et al., 2017a).

Another function of snoRNAs is to be the precursors of some miRNAs (Scott and Ono, 2011). The role for miRNAs in cells is generally that to downregulate or eliminate the expression of specific cytoplasmic mRNAs, but no identified action on tail regeneration has been discovered so far. In our transcriptome study (Vitulo et al., 2017a), we only detected five upregulated miRNAs that are expressed in both regenerating tail and limbs, indicated as *mi*1-5 in Figure 9F, whereas no exclusive miRNAs of the regenerating tail or limb were found in our analysis. However, differently from snoRNAs, most miRNAs are much more highly expressed in the regenerating limb blastema at 16–18 days in comparison to the tail blastema at 12–14 days (Fig. 9F). These findings indicate that these miRNAs are involved in inflammation and in differentiating processes such as fibrosis and osteogenesis that are absent in the regenerating blastema at the analyzed stages (Barber, '44; Alibardi, 2016a,c). Also the three miRNAs uniquely detected in the apical regions of the

regenerating tail in *A. carolinensis* (Hutchins et al., 2016) are likely associated with the differentiation of muscle and cartilaginous tissues (McCarthy, 2008; Zhao et al., 2013), although a definite conclusion on the role of miRNAs for organ regeneration in lizards demands further investigation.

DOWNREGULATED GENES SUGGEST THAT THE TAIL BLASTEMA IS IMMUNOSUPPRESSED

The analysis of up- and downregulated genes of tail and limb blastemas reveal profound differences between the two organs, indicating that in the tail the key genes (sno/miRNAs and signaling) can be expressed in a permissive, immunosuppressed environment, absent in the limb (Vitulo et al., 2017a,b). Since the histological composition of the normal tail and limb is different from their respective regenerating blastemas, we expect that gene expression in mature, physiologically active tissues is very different in comparison to the regenerating tail or the scarring limbs. A strong downregulation of genes operating in the normal, physiological activity of differentiated cells is present in the regenerating tail and limb blastemas. The genes regulating the activity of the cytoskeleton, channel proteins for the movement of water and ions in cells, and for numerous enzymes of the metabolism of differentiated cells, are also strongly downregulated in both tail and limb blastemas. Active genes of differentiated tissues however are not important for triggering organ regeneration, whereas other downregulated genes, important for allowing organ regeneration, have been identified.

Among the latter, numerous genes of the immune system, for both the humoral (*Ighv, jcain, Igeva* etc.) and cellular (*mchII, perf, lcp2* etc) responses, are strongly downregulated (Fig. 10A). The study indicates that the tail blastema is a temporary immune-tolerated, embryonic-like organ connected to an adult body, and therefore no rejection occurs and no inflammation gives rise to a scar. Among other mechanisms, a process of activation of immune-tolerant macrophages (healing or type M2) may occur during early stages of regeneration and makes the blastema a temporary immune-evasive organ, like in cancer (Gabrilovich et al., 2012). This hypothesized mechanism of immunological tolerance activated in the tail blastema that is connected with the remaining body of the lizard remains an hypothesis that has to be verified in molecular terms. It therefore appears that lizards (like amphibians) more than possessing unique "regenerative genes" hypothetically missing also in other amniotes cannot express genes triggering organ regeneration mainly due to the interference from the immune system that impedes any form of organ-formative recapitulation (Alibardi, 2017a). The latest results therefore suggest that the depression of immune genes is one of the main reasons why the tail can regenerate despite the connection with the circulating immune cells derived from the rest of the body. This condition may be temporary, but, once the blastema is formed, the apical region is responsible for Wnt-signaling proteins and snoRNAs, is somehow tolerated for the entire period required to reform a new tail (1–2 months; Figs. 8A–C and 10A–C). Immunolabeling for Wnt1 indicates that the blastema contains a protein of 35 and 45 kDa, especially located in the ependymal and wound epidermis (Fig. 10C), confirming the transcriptome data (Alibardi, 2017b). Circulating lymphocytes and extra-vasating macrophages are continuously permeating the blastema cone, but they do not attach these embryonic tissues, at least in the first or second regeneration. As opposed, immunoglobulins in the serum and large lymphoblasts in the circulating blood increases after a third and fourth consecutive amputation of the tail, or in lizards constantly maintained at high and constant "endothermic" temperatures (37–40°C), when the tail also tends to form scars (Alibardi, 2014).

Differently from the tail, in the limb the forming blastema and the wound epidermis are invaded by immune cells during the initial 2–3 weeks of regeneration, and an AEP is not formed so impeding limb regeneration (Alibardi and Toni, 2005; Alibardi, 2010b), a process similar to those described in anuran amphibians before metamorphosis (Mesher and Neff, 2003; Mescher et al., 2013, 2016; Godwin and Rosenthal, 2014; Alibardi, 2017a; Figs. 1C and 5). The study on downregulated genes in the scarring limb shows no inhibition of immune genes but instead a general reduction in the expression of myofunctional genes for numerous myosins, troponins, tropomyosins, and of cell-functional genes coding for dyneins, kynesins, ion and metabolite carriers, and for cytoskeletal proteins (Fig. 10D). It is likely that, after injection of FGF to stimulate limb regeneration, inflammation is reduced and the apical region of the outgrowth becomes temporary similar to that of a regenerating tail, with proliferating cells mainly located at the tip (Figs. 7E and 2015a). Further study is needed to determine whether Wnt and other signaling proteins, and noncoding RNAs, are upregulated in induced limb outgrowths, in the attempt to stimulate limb regeneration in lizards.

EVOLUTIONARY CONSIDERATIONS AND CONCLUSIONS

The present study indicates that amniote genomes likely contain "regeneration genes," similar to those in amphibians, but they are faced with problems generated by their efficient immune system that does not accept as self-dedifferentiated cells derived from injured or amputated organs, cells that are required to recreate an embryonic organ for regeneration. Future researchers in this area will focus on key genes (noncoding RNAs, Wnt, and related genes and immune genes) to determine how the regenerating tail of lizards manages to escape inflammation and the immunological control that instead directs the limb and other organs to repair with a scarring process (Ferguson and O'Kane, 2004). The balanced equilibrium that normally allows tail regeneration is altered by experimental interventions on the tail blastema such as wounding, AEP removal or cauterization, treatment with toxic/irritating ions such as Be and

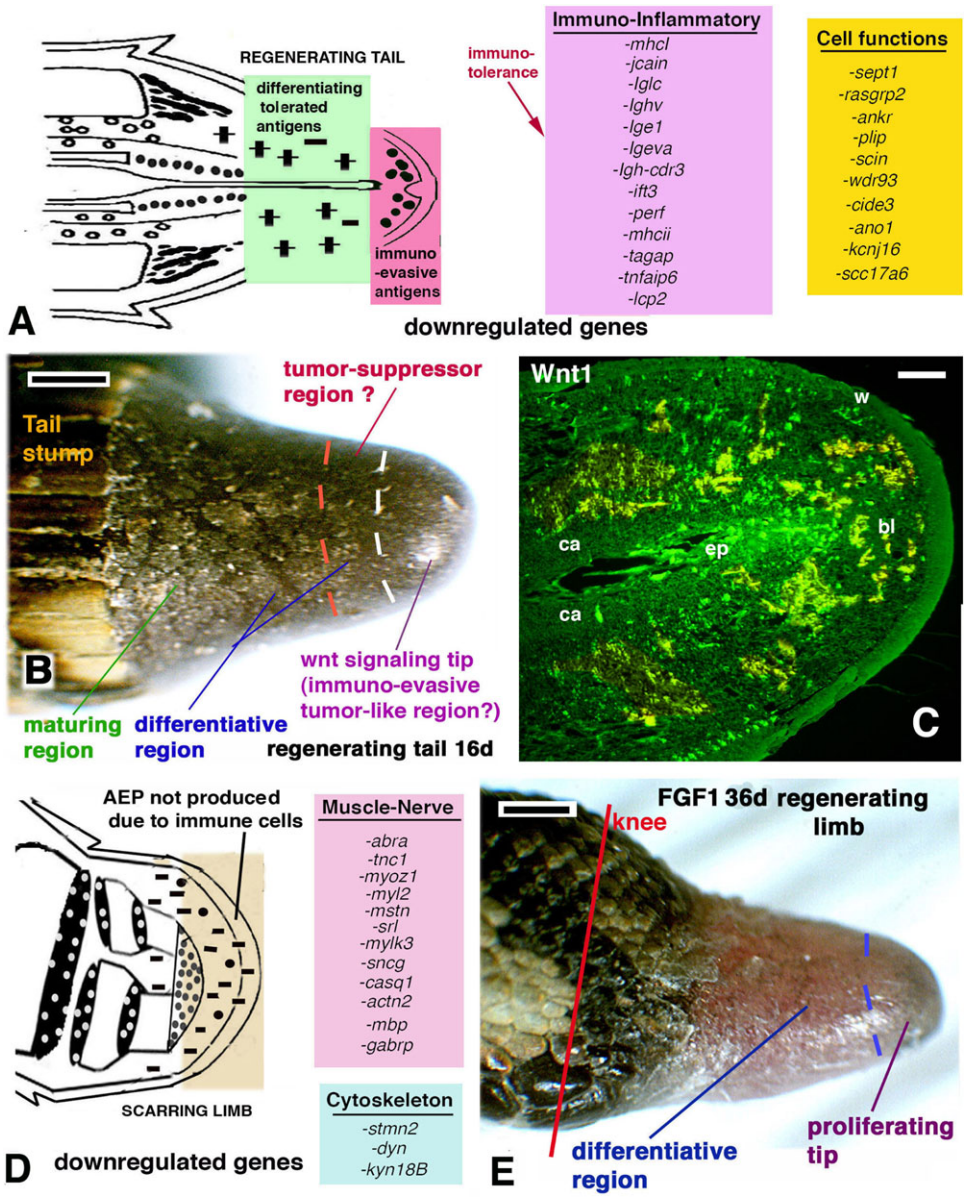

Figure 10. Regenerating tail (A–C) and limb (D and E) with some downregulated genes indicated. (A) Schematic drawing showing some downregulated genes (colored columns), expressed in the regenerating tail (the proliferating apical region is in red and the proximal differentiating region is in green). The red arrow points to a list of immune genes coding for immunoglobulins and T-cells markers. (B) Elongating cone indicated by dashes the hypothetical proximal-distal, embryonic-like zones. Bar, 1 mm. (C) is an immunofluorescent section of the apical tip of a regenerating tail showing the green immunofluorescence for the Wnt1-protein (bl, blastema; ca, regenerating cartilaginous tube; ep, apical ependymal; w, wound epidermis). Bar, 50 μm. (D) Schematic drawing of the scarring limb showing some downregulated genes of the muscle-nerve and cytoskeleton categories. (E) Stimulated hindlimb outgrowth after FGF1 treatment, resembling an elongated tail cone, where the apical proliferating tip (dashes) may have similar proliferative process like in the tail. The red line indicates the level of the knee. Bar, 1 mm. [Color figure can be viewed at wileyonlinelibrary.com]

Cd, repetitive tail amputations (Alibardi, 2014), as for amphibians (Mescher et al., 2013). These different manipulations stimulate the latent inflammatory-immune tendency of lizards, and in general of amniotes, to remove embryonic antigens as they do with those of mutated or tumor cells, one of the main reasons for the evolution of the adaptive immune system (Danilova, 2006). This suggests that adaptive immunity is one of the main obstacles to tissue regeneration for higher vertebrates. Among amniotes, only lizards have evolved a mechanism that compromises the two processes, cell proliferation and tissue patterning without degenerating into an uncontrolled tumorigenic process, and allowing tail regeneration in an immune-tolerated condition. We summarize here some hypotheses on the peculiar conditions involved in the evolution of successful regeneration in lizard's tail (Alibardi, 2010a): (1) ectothermic amniotes with an immune system less efficient than in endotherms, at least below 25°C, (2) the presence of self-amputation planes and potent antimicrobial peptides effective below 25°C that limit inflammation and consequently scarring, (3) the presence of sparse stem cells and of a dedifferentiating process that allows the formation of an apical blastema, (4) temporary immune-suppression (immune-evasion) of the embryonic antigens formed in the regenerative blastema, and (5) formation of an AEP that maintains the growth of the apical mesenchyme, leading to organ elongation.

In addition to summarizing research carried out over the past 30 years, one of the main goals of the present study was to draw the attention of modern developmental biologists and immunopathologists to the lizard model as a unique opportunity to understand the biological limits of healing in amniotes, including mammals. Current information on the genes activated in different regions of the same body in lizard (tail or limb) are a unique gift that nature offers to investigators to connect the amazing regenerative capability of anamniotes equipped with a weak immune system to the limitations imposed to the amniote condition, largely by their immune system. This knowledge will foster the understanding of the link between regeneration and scarring, and the attempts to induce the regeneration of the autopodium in lizards, a first step to move to other amniotes, including humans. During development, the different organs develop with an absent or immature immune system, and if we want to make some organ regeneration possible we have to recreate embryonic conditions, in particular an environment similar to that present during embryogenesis, which is immune-free, a recapitulation of development that also occurs in immune-depressed conditions.

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